Tumor Necrosis Factor Alpha Gene Polymorphism Associated with Increased Susceptibility to Venous Leg Ulceration

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TO THE EDITOR

Venous ulcers represent the severe end of the spectrum of chronic venous disorders of the leg. Prevalence of chronic venous ulceration increases progressively with age, with a point prevalence of approximately 1% in the population over the age of 70 years (Baker *et al.*, 1991; Nelzen *et al.*, 1994; O'Brien *et al.*, 2000). Primary risk factors for venous ulcers are those for the development of chronic venous insufficiency, namely primary abnormalities of the venous wall and valves and secondary changes owing to previous venous thrombosis (Nicolaides, 2000).

Importantly, not all individuals with venous insufficiency develop leg ulcers. It is likely that a number of inherent factors contribute to susceptibility, including inherited genetic alterations. Apart from the risk factors for chronic venous insufficiency, increasing age and female gender are the only other established risk factors for the development of venous leg ulcers (Graham *et al.*, 2003). Evidence for the role of genetic predisposition is limited, but the association of a single nucleotide polymorphism (SNP) in the 3′ untranslated region of the fibroblast growth factor receptor-2 (FGFR-2) gene with venous leg ulceration has recently been reported in this journal (Nagy *et al.*, 2005).

There is interest in the role of inflammatory processes in the pathogenesis of venous ulceration. We have found that leg ulcer wound fluid levels of the proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) are much higher than the levels found in wound fluid from normal acute wounds (Baker and Leaper, 2000), and decrease as ulcers start to heal (Wallace and Stacey, 1998). Other studies have shown that levels of matrix metalloproteinases (MMPs), including MMP-3 (Vaalamo *et al.*, 1996) and MMP-2 (Wysocki *et al.*, 1993), are elevated in venous ulcers. We hypothesized that functional polymorphisms in proinflammatory cytokine and MMP genes, or genes involved in their regulation, may be risk factors for the development of venous leg ulcers.

We conducted a case–control study comparing the frequency of gene polymorphisms in 181 Caucasian patients with a history of confirmed venous leg ulceration and 181 age- and gender-matched healthy controls, also of Caucasian background. Control subjects were excluded if they had abnormal venous function (venous refilling time less than 25 seconds on photoplethysmography). Subjects were excluded if they had type I diabetes or rheumatoid arthritis. All subjects provided their written informed consent. The study was approved by the Fremantle Hospital Human Research Ethics Committee and was conducted according to the Declaration of Helsinki Principles.

Polymorphisms in genes playing a role in normal wound healing or in the regulation of inflammatory processes were selected (Table 1). They included a G→A polymorphism in the promoter of the TNFA gene (*TNFA-308*) located in the central region of the major histocompatibility complex (MHC). This is a region of strong linkage disequilibrium, where large conserved blocks of genes are maintained in particular arrangements of alleles known as ancestral haplotypes. Approximately 70% of Caucasian individuals carrying the *TNFA−308A* allele also have part or all of the 8.1 ancestral haplotype (HLA-A1, B8, DR3, and DQ2) associated with numerous immunopathological disorders, including type I diabetes and rheumatoid arthritis (Price *et al.*, 1999). We therefore also examined a polymorphism located in intron 10 of the HLA-B-associated transcript-1 (BAT-1) gene used as a marker of this haplotype (Price *et al.*, 2002). Polymorphisms were genotyped using polymerase chain reaction (PCR)-based methods (Table 1). The estimation of relative risk of venous ulceration is based on logistic regression analysis. Crude and adjusted (for age and gender) odds ratios (OR) are reported with 95% confidence intervals (95% CI). Analyses were performed with the statistical package SPSS for Windows version 11.5.0.

Carriage of the *TNFA-308A* allele was a significant risk factor for venous ulceration (adjusted OR 2.48; 95% CI 1.54–3.97; $P=0.000155$) (Table 2). Although not statistically significant, the risk appeared to be higher for *TNFA−308AA* homozygotes (adjusted OR 8.08; 95% CI 0.95–68.53) than *TNFA−308GA* heterozygotes (adjusted OR 2.32; 95% CI 1.44–3.76) compared to the wild type (GG) (Table 3). The marker in intron 10 of the BAT1 gene (C insertion) was also a significant risk factor (adjusted OR 2.00; 95% CI 1.16–3.44) (Table 2). Cases and controls were evenly matched for age and

Abbreviations: BAT1, HLA-B-associated transcript 1; CI, confidence interval; FGFR, fibroblast growth factor receptor; F-SSCP, fluorescence-based single strand conformational polymorphism; HLA, human leukocyte antigen complex; IL-1, interleukin-1; IL-1RN, interleukin-1 receptor antagonist; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; OR, odds ratio; PAI1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TNFα, tumor necrosis factor alpha; TNFA, tumor necrosis factor alpha (gene)

Table 1. Genotyping methods and disease association references

Gene (polymorphism)	Primer/probe sequences	Genotyping method	AT[1] (°C)	Disease associations
TNFA (−308 G/A)	F: 5′-CCTGCATCCTGTCTGGAAGT	Fluorescence-based single strand conformation polymorphism analysis (F-SSCP) (Grieu *et al.*, 2004)	55	Hajeer and Hutchinson (2001)
	R: 5′-CTTCTGGGCCACTGACTGAT			
	Designed using Primer3 software (Rozen and Skaletsky, 2000)			
MMP3 (−1171 5A/6A)	F: 5′-TGGTTCTCCATTCCTTTGATG	F-SSCP analysis (Grieu *et al.*, 2004)	55	Ye (2000)
	R: 5′-AATTCACATCACTGCCACCA			
	Designed using Primer3 software (Rozen and Skaletsky, 2000)			
PAI1 (−675 4G/5G)	F: 5′-CTCAGGGGCACAGAGAGAGT	F-SSCP analysis (Grieu *et al.*, 2004)	55	Nordt *et al.* (2001)
	R: 5′-TCTTGGTCTTTCCCTCATCC			
	Designed using Primer3 software (Rozen and Skaletsky, 2000)			
IL-1RN intron 2 (variable number of tandem repeats: VNTR)	F: 5′-CTCAGCAACACTCCTAT	Agarose gel fragment size analysis (3% agarose)	50	Witkin *et al.* (2002)
	R: 5′-TCCTGGTCTGCAGGTAA			
	(Tarlow *et al.*, 1993)			
MMP2 (−1306 C/T)	F: 5′-GCCATTGTCAATGTTCCCTAAAACA	TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA)	60	Price *et al.* (2001)
	R: 5′-TGACTTCTGAGCTGAGACCTGAA			
	Probe FAM: 5′-CTAAAGAGGTAGAGTGCT			
	Probe VIC: 5′-CTAAAGAGGTGGAGTGCT			
	Assay-By-Design℠ (Applied Biosystems, Foster City, CA)			
BAT1 intron 10 (−/C)	F: 5′-CTACCGTGTCTGTTCAACTGAGAA	TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA)	60	Price *et al.* (1999)
	R: 5′-GCCTCACTTTCCCCTTTTCATG			
	Probe FAM: 5′-TTATTCTGACCATGCTACG			
	Probe VIC: 5′-TTCTGACCATGGCTACG			
	Assay-By-Design℠ (Applied Biosystems, Foster City, CA)			

BAT1=HLA-B-associated transcript 1; IL-1RN=interleukin-1 receptor antagonist; MMP=matrix metalloproteinase; PAI1=plasminogen activator inhibitor 1; TNFA=tumor necrosis factorα (gene).
[1]Annealing temperature.

gender, and adjustment of the odds ratios for these covariates had little effect. Polymorphisms in the MMP2, MMP3, PAI1, and IL-1RN genes were not associated with ulceration in this population. All genotypes were in Hardy–Weinberg equilibrium.

This study focused on genetic associations to test the hypotheses about causal pathways for venous leg ulceration. The use of case–control studies to identify disease-associated genes carries a high risk of false-positive findings, and we calculated the false-positive report probability (FPRP) using an approach recently described by Wacholder *et al.* (2004). For carriage of *TNFA−308A*, the FPRP varied from 0.007 to 0.076 for prior probabilities of the hypothesis ranging from 0.1 (high) to 0.01 (moderate), well below the 0.2 level proposed by Wacholder *et al.* (2004) as stringent. For the BAT1 intron 10 marker (C insertion), the FPRP was also below 0.2 (0.181) using a prior probability of 0.1. This analysis suggests that the *TNFA-308A* association is

Table 2. Results of genotype analysis

Gene (polymorphism[2])	High-risk allele: carrier frequency[1] (%) Ulcer patients	Controls	Odds ratio (95% CI) Significance	Adjusted OR[3] (95% CI) Significance
TNFA (−308 G/**A**)	43.1	22.6	2.59 (1.64–4.08)	2.48 (1.54–3.97)
			P=0.00003	*P*=0.000155
BAT1 (−/**C** in intron 10)	28.8	16.3	2.08 (1.23–3.52)	2.00 (1.16–3.44)
			P=0.0055	*P*=0.012
MMP3 (−1171 **5A**/6A)	73.5	72.4	1.00	1.11
			(0.62–1.60)	(0.68–1.80)
			NS	NS
MMP2 (−1306 **C**/T)	93.2	92.9	1.08 (0.46–2.51)	1.17 (0.47–2.92)
			NS	NS
PAI1 (–675 **4G**/5G)	82.3	79.5	1.20 (0.71–2.02)	1.11 (0.64–1.92)
			NS	NS
IL-1RN (variable number of tandem repeats in intron 2. ***IL-1RN*2*** contains two repeats)	49.4	44	1.24 (0.81–1.91)	1.31 (0.84–2.05)
			NS	NS

BAT1=HLA-B-associated transcript 1; CI=confidence interval; IL-1RN=interleukin-1 receptor antagonist; MMP=matrix metalloproteinase; OR=odds ratio; PAI1=plasminogen activator inhibitor 1; TNFA=tumor necrosis factorα (gene).
[1]Carriers include both heterozygotes and homozygotes.
[2]Bold type indicates postulated high-risk allele.
[3]Adjusted for age and gender. NS=not significant ($P>0.05$).

Table 3. *TNFA−308* genotype results

TNFA−308 genotype[2]	Frequency[1] Ulcer patients	Controls	Adjusted OR (95% CI)[3]	Significance
GG	103 (56.9)	140 (77.3)	Reference	
G**A**	71 (39.2)	40 (22.1)	2.32 (1.44–3.76)	*P*=0.001
AA	7 (3.9)	1 (0.06)	8.08 (0.95–68.5)	*P*=0.055

CI=confidence interval; OR=odds ratio; *TNFA*=tumor necrosis factorα (gene).
[1]Data show numbers of subjects with percentages in parentheses.
[2]Bold type indicates postulated high-risk allele.
[3]Adjusted for age and gender.

particularly ''noteworthy'', with a low risk of being a false-positive finding.

The approximate two-fold increase in risk of ulceration seen in carriers of the *TNFA−308A* allele or the *BAT1* marker of the 8.1 MHC ancestral haplotype is likely to be biologically significant. The association found here was independent of the presence of type I diabetes or rheumatoid arthritis. The *TNFA−308A* polymorphism has been shown to increase TNFα protein levels in some studies (Hajeer and Hutchinson, 2001). We have inferred that the 8.1 ancestral haplotype of the MHC was carried by 66% of the *TNFA−308A* individuals in the study through the *BAT1* marker (data not shown). Several candidate genes in the MHC region have the potential to modulate immune or inflammatory responses. It is not possible to conclude from this data whether the association with venous ulceration is owing to the direct influence of the TNFA−308A allele or owing to linkage disequilibrium with another polymorphism in this region of the MHC. The sample size of the study was too small to allow subgroup analysis.

A larger population-based study looking at an increased number of genetic markers is required to identify whether the causal allele for increased risk of venous ulceration in this region of the MHC is TNFA−308A. Future investigations on the effect of TNFA genotype on local TNFα mRNA and protein levels in leg ulcers will help to determine if the TNFA−308A polymorphism has a role in the disease process. These results support the hy-

pothesis that chronic inflammation is an important part of the pathophysiology of venous ulceration. If future results provide evidence that TNFA−308A is a critical allele responsible for increased risk, specific treatments directed at reducing TNFα levels may be appropriate. In addition, the TNFA−308A allele may be a useful marker to identify patients at high risk of ulceration among those who are already at significant risk: in particular, patients with a history of deep vein thrombosis.

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REFERENCES

Baker EA, Leaper DJ (2000) Proteinases, their inhibitors, and cytokine profiles in acute wound fluid. *Wound Rep Reg* 8:392–8

Baker SR, Stacey MC, Jopp-McKay A, Hoskin SE, Thompson PJ (1991) Epidemiology of chronic venous ulcers. *Br J Surg* 78:864–7

Graham ID, Harrison MB, Nelson EA, Lorimer K, Fisher A (2003) Prevalence of lower-limb ulceration: a systematic review of prevalence studies. *Adv Skin Wound Care* 16:305–16

Grieu F, Joseph D, Norman P, Iacopetta B (2004) Development of a rapid genotyping method for single nucleotide polymorphisms and its application in cancer studies. *Oncol Rep* 11:510–4

Hajeer AH, Hutchinson IV (2001) Influence of TNFα gene polymorphisms on TNFα production and disease. *Hum Immunol* 62:1191–9

Nagy J, Szolnoky G, Szabad G, Bata-Csörgo Z, Dobozy A, Kemény L *et al.* (2005) Single nucleotide polymorphisms of the fibroblast growth factor receptor 2 gene in patients with chronic venous insufficiency with leg ulcer. *J Investig Dermatol* 124:1085–8

Nelzen O, Bergqvist D, Lindhagen A (1994) Venous and non-venous leg ulcers: clinical history and appearance in a population study. *Br J Surg* 81:182–7

Nicolaides AN (2000) Investigation of chronic venous insufficiency. *Circulation* 102: e126–63

Nordt TK, Lohrmann J, Bode C (2001) Regulation of PAI-1 expression by genetic polymorphisms – impact on atherogenesis. *Thromb Res* 103:S1–5

O'Brien JF, Grace PA, Perry IJ, Burke PE (2000) Prevalence and aetiology of leg ulcers in Ireland. *Ir J Med Sci* 169:110–2

Price P, Bolitho P, Jaye A, Glasson M, Yindom LM, Sirugo G *et al.* (2002) A Gambian TNF haplotype matches the European HLA-A1,B8,DR3 and Chinese HLA-A33,B58,DR3 haplotypes. *Tissue Antigens* 62:72–5

Price P, Witt C, Allcock R, Sayer D, Garlepp M, Kok CC *et al.* (1999) The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological disorders. *Immunol Rev* 167: 257–74

Price SJ, Greaves DR, Watkins H (2001) Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene. *J Biol Chem* 276:7549–58

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics methods and protocols.* (Krawetz S, Misener S, eds), Totowa, NJ: Humana Press, 365–86

Tarlow JK, Blakemore AI, Lennard A, Solari R, Hughes HN, Steinkasserer A *et al.* (1993) Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Hum Genet* 91:403–4

Vaalamo M, Weckroth M, Puolakkainen P, Kere J, Saarinen P, Lauharanta J *et al.* (1996) Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. *Br J Dermatol* 135:52–9

Wacholder A, Chanock S, Garcia-Closas M, El ghormli L, Rothman N (2004) Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 96:434–42

Wallace HJ, Stacey MC (1998) Levels of tumour necrosis factor-alpha (TNF-alpha) and soluble TNF receptors in chronic venous leg ulcers – correlations to healing status. *J Investig Dermatol* 110:292–6

Witkin SS, Gerber S, Ledger WJ (2002) Influence of interleukin-1 receptor antagonist gene polymorphism on disease. *Clin Infect Dis* 34:204–9

Wysocki AB, Staiano-Coico L, Grinnell F (1993) Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Investig Dermatol* 101:64–8

Ye S (2000) Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. *Matrix Biol* 19:623–9